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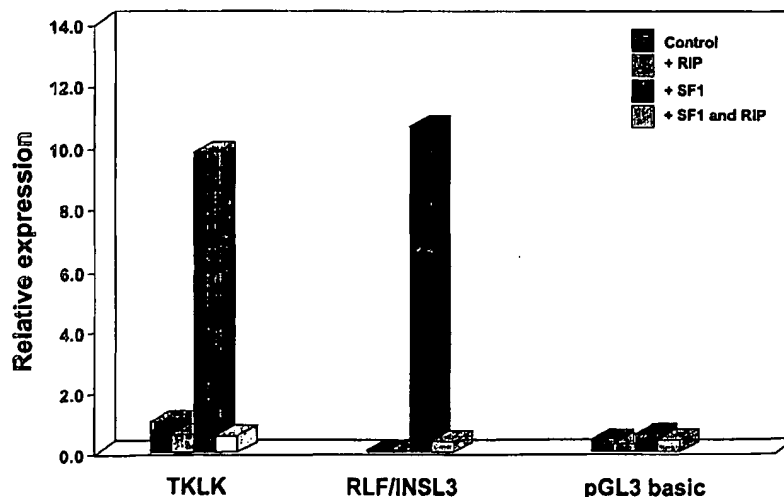
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(54) Title: SCREENING FOR ANTI-OVULATORY COMPOUNDS



(57) Abstract: Screening methods for identifying compounds that are useful as contraceptives are provided. In particular, the compounds inhibit the interaction between steroidogenic factor 1 (SF-1) and nuclear receptor interacting protein (Nrip1) or the up-regulate SF-1 expression.

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SCREENING FOR ANTI-OVULATORY COMPOUNDS

All documents cited herein are incorporated by reference in their entirety

TECHNICAL FIELD

This invention relates to screening methods for identifying compounds that are useful as
5 contraceptives. In particular, the invention provides methods of screening for compounds
that inhibit the interaction between steroidogenic factor 1 (SF-1) and nuclear receptor
interacting protein 1 (Nrip1). The invention also provides methods of screening for
compounds that promote expression of SF-1. Preferably, the compounds identified by the
methods of the invention promote the transcription of SF-1 regulated genes.

10 BACKGROUND ART

Oral contraceptives containing a combination of a progesterone component and an
oestrogen component have been used since the 1960's. The progesterone component of the
combined pill inhibits ovulation by suppressing luteinising hormone. The oestrogen
component acts to inhibit follicle stimulating hormone and luteinising hormone to promote
15 the ovulation inhibitory effect of the progesterone.

These contraceptives work by increasing oestrogen and progesterone levels to suppress the
levels of other hormones in the body, so the use of these oral contraceptives is associated
with artificial changes in hormone levels. It is possible that these changes may have
adverse side-effects. For example, oral contraceptives have been associated with increased
20 risk for myocardial infarction, stroke, venous thromboembolism and possibly breast cancer
(Pymar & Crenin, 2001, *Semin Reprod Med* 19(4): 309-12). There is therefore a need to
identify compounds that block ovulation without altering hormone levels. Thus, an object
of the invention is the provision of a method for identifying alternative compounds that
will inhibit ovulation in mammals.

25 The nuclear receptor corepressor nuclear receptor interacting protein 1 (Nrip1), also known
as RIP140, is known to be essential for female fertility. Female mice null for Nrip1 are
viable but infertile because of complete failure to release oocytes at ovulation (White *et al*,
2000, *Nature Medicine*, 6:1368-1374). Nrip1 is thought to have a secondary role in
maintenance of pregnancy (Leonardsson *et al*, 2002, *Endocrinology*, 143(2): 700-707).
30 Prior to the discovery of its role in ovulation, Nrip1 was known to act as a co-activator or
co-repressor of a large number of different nuclear hormone receptors. More recently it has
been found to modulate transcription of the steroidogenic acute regulatory protein gene
(StAR) through interactions with the transcription factors steroidogenic factor 1 (SF-1; also

know as Ad4BP) and DAX-1 (Sugawara *et al*, 2001, *Endocrinology* 142: 3570-3577). StAR is involved in adrenal gonadal steroid synthesis. However, SF-1 has not been implicated in the control of ovulation.

DISCLOSURE OF THE INVENTION

5 It has now been discovered that SF-1 up-regulates the transcription of various genes and that this SF-1 dependent transcription is inhibited in the presence of Nrip1. It is therefore postulated that the absence of ovulation previously seen in Nrip1 null female mice is a result of gene transcription by SF-1 in the absence of any inhibition by Nrip1. Accordingly, it is postulated that promotion of transcription of SF-1 regulated genes, either by inhibiting
10 the interaction between SF-1 and Nrip1 or by promoting expression of SF-1, will inhibit ovulation.

As a first method, the invention therefore provides a method of screening for compounds that inhibit the interaction between SF-1 and Nrip1, said method comprising assessing inhibition of the interaction between SF-1 and Nrip1 in the presence of a candidate
15 compound. Candidate compounds that inhibit the interaction between SF-1 and Nrip1 may have contraceptive efficacy.

Preferably, the method of screening for compounds that inhibit the interaction between SF-1 and Nrip1 comprises the steps of:

- (a) mixing Nrip1, SF-1 and one or more candidate compounds;
- 20 (b) incubating the mixture to allow Nrip1, SF-1 and the candidate compound(s) to interact; and
- (c) assessing whether the interaction between Nrip1 and SF-1 is inhibited.

The mixing of Nrip1, SF-1 and candidate compound in step (a) may be done in any order.

As a second method, the invention provides a method of screening for compounds that
25 promote the expression of SF-1, said method comprising assessing the level of expression of SF-1 in the presence of a candidate compound. Candidate compounds that promote the expression of SF-1 may override the inhibition of SF-1 by Nrip1 and hence have contraceptive efficacy.

Direct screening for inhibitors of SF-1:Nrip1 interaction:

Inhibition of the SF-1/Nrip1 interaction in the presence of candidate compounds may be assessed directly. Various methods for direct detection of protein/protein interactions are available.

For example, one or both of SF-1 and Nrip1 may be labelled with a fluorescent label such that the interaction between SF-1 and Nrip1 may be detected by an intrinsic fluorescence change which occurs when an SF-1:Nrip1 complex is formed or disrupted. For example, SF-1 may be joined to a fluorescence resonance energy transfer (FRET) donor and Nrip1 to a FRET acceptor (or *vice versa*) such that, when SF-1 and Nrip1 interact, stimulation of the FRET donor excites the FRET acceptor causing it to emit photons. Interaction may be also be detected by fluorescent labelling of SF-1 and/or Nrip1 such that fluorescence is quenched when they form a complex.

Other methods for assessing interaction between SF-1 and Nrip1 may include using NMR to determine whether an SF-1:Nrip1 complex is present when a candidate compound is present.

The presence of a SF-1:Nrip1 complex may also be detected as a band at a particular position when run on a gel. Disruption of the complex by addition of a candidate compound may be detected by the presence of bands at different positions on the gel.

Interaction of Nrip1 and SF-1 may also be assessed by detecting the accessibility of peptide sequences (*e.g.* epitopes) on Nrip1 and/or SF-1 that are masked when the two proteins form a complex. For example, motifs on Nrip1 that interact with nuclear receptors have been identified in WO98/49561, Lee *et al* (Mol Cell Biol, 1998, 18(11): 6745-44) and in Wei *et al* (J. Biol. Chem., 2001, 276(19): 16107-12). A lack of interaction between Nrip1 and SF-1 may therefore be determined by detection of such motifs, for example, using antibodies.

Another method of assessing whether interaction between Nrip1 and SF-1 is inhibited by a candidate compound may involve immobilising Nrip1 on a solid surface and assaying for the presence of free SF-1 in the presence of SF-1 and a candidate compound. If interaction between SF-1 and Nrip1 is inhibited by the candidate compound, free SF-1 will be detected. The SF-1 may be labelled to facilitate detection. This type of assay may also be carried with SF-1 being immobilised on the solid surface. Interaction between the immobilised and the free protein may also be monitored by a process such as surface plasmon resonance.

Other methods are described by Sugawara *et al*, 2001, *Endocrinology* 142: 3570-3577.

Indirect screening for inhibitors of SF-1:Nrip1 interaction using two-hybrid systems:

Whether the interaction between SF-1 and Nrip1 is inhibited in the presence of a candidate compound may also be assessed indirectly. One indirect method of screening for inhibition
5 of the interaction between SF-1 and Nrip1 in the presence of a candidate compound involves using a two-hybrid system. SF-1 may be fused to an activation domain of a transcription factor and Nrip1 to a DNA-binding domain of a transcription factor (or *vice versa*), such that interaction between SF-1 and Nrip1 promotes the transcription of a reporter gene in a cell.

- 10 The invention provides a method of screening for compounds that inhibit the interaction between SF-1 and Nrip1, said method comprising:

- a) contacting a cell containing a nucleic acid molecule comprising a promoter operatively linked to a reporter gene with: (i) a first fusion protein comprising one of SF-1 and Nrip1 fused to the activation domain of a transcription factor, (ii) a
15 second fusion protein comprising the other of SF-1 and Nrip1 fused to the DNA-binding domain of a transcription factor; and (iii) a candidate compound; and
- b) assessing the level of expression of the reporter gene,

wherein interaction between SF-1 and Nrip1 promotes transcription of the reporter gene by activating said promoter.

- 20 This method may be used to assess interaction between Nrip1 and SF-1 in any eukaryotic cell. Preferably, the method is used to assess the interaction between Nrip1 and SF-1 in a yeast cell or a mammalian cell. Where the candidate compound is an organic compound and a yeast two-hybrid system is being used, the permeability of the yeast cell wall is preferably enhanced *e.g.* by using chemicals such as polymyxin B.
- 25 The level of expression of a reporter gene in the two-hybrid system is indicative of the level of interaction between SF-1 and Nrip1. A candidate compound that inhibits the interaction between SF-1 and Nrip1 decreases or abolishes the level of expression of the reporter gene.

- Preferably, the reporter gene is easily assayed. For example, the reporter gene may give a
30 detectable signal, such as a visible signal. The reporter gene may encode a protein which gives a visible signal itself, or which catalyses a reaction which gives a visible change *e.g.* a fluorescent protein or an enzyme. The reporter gene may encode an enzyme such as a

beta-galactosidase or a peroxidase, both of which are commonly used with coloured substrates and/or products. The reporter gene may encode a green fluorescent protein (GFP) or a fluorescent derivative thereof such as YFP or CFP (see Prasher *et al*, 1995, *Trends Genet* 11(8): 320). The reporter gene may encode a luminescent protein, such as
5 luciferase.

The reporter gene may drive DNA replication (Vasavada *et al*, 1991, *PNAS*, 88:10686-10690) in the cell or may encode a drug resistance marker (Fearon *et al*, 1992, *PNAS* 89: 7958-7962).

The reporter gene may encode a protein that enables positive selection of cells in which the
10 interaction between SF-1 and Nrip1 is inhibited. For example, the reporter gene may encode a protein that is toxic or cytostatic so that only cells that do not express the protein are able to survive or grow. As a result, the only cells to survive are those in which the candidate compound inhibits the interaction between SF-1 and Nrip1 so that the reporter gene is not expressed. Examples of reporter genes of this type that may be used in yeast
15 include URA3, LYS2 and CYH2 (see Vidal *et al*, 1996, *PNAS*, 93: 10315-10320). The protein encoded by the reporter gene may also prevent cell growth in the absence or presence of a particular amino acid or other component in cell media. For example, the reporter gene may encode a DNA-binding protein, Tn10 tetracycline, which represses transcription of a TetRop-HIS3 gene so that yeast cells in which the reporter gene is
20 expressed do not grow in the absence of histidine (see Shih *et al*, 1996, *PNAS*, 93: 13896-13901). In contrast, yeast cells in which the interaction between Nrip1 and SF-1 has been disrupted do not express TN10 tetracycline and are consequently able to grow in the absence of histidine.

The proteins encoded by the reporter genes may be in the form of fusion proteins. Methods for the generation of fusion proteins are standard in the art and will be known to the skilled reader. For example, most general molecular biology, microbiology recombinant DNA technology and immunological techniques can be found in Sambrook *et al.*, (Molecular Cloning, A Laboratory Manual, Cold Harbor-Laboratory Press, Cold Spring Harbor, N.Y., 2000) or Ausubel *et al.*, (Current Protocols in Molecular Biology, Wiley Interscience, NY, 1991).

Other indirect screening methods for inhibitors of SF-1:Nrip1 interaction:

Because SF-1 is a transcription factor which is inhibited by Nrip1, interaction between SF-1 and Nrip1 may also be assessed indirectly by means of a reporter gene under the control of a promoter which is regulated by SF-1. The invention provides a method of screening for compounds that inhibit the interaction between SF-1 and Nrip1, said method comprising:

- a) contacting a nucleic acid molecule, comprising an SF-1-regulated promoter operatively linked to a reporter gene, with one or more candidate compounds, in the presence of SF-1 and Nrip1; and
- b) assessing the level of expression of the reporter gene.

This method employs a nucleic acid molecule comprising a promoter operatively linked to a reporter gene, such that transcription of the reporter gene is under the control of the promoter. These nucleic acid molecules are referred to as reporter constructs. Such reporter constructs are already known from the prior art *e.g.* Koskimies *et al.* (2002) *Endocrinology* 143:909-919 describes reporter constructs which express luciferase under the control of a RLF promoter, with transcription regulated by SF-1.

The promoter in the construct is an SF-1 regulated promoter from which SF-1 is able to promote transcription of the reporter gene. SF-1 regulated promoters contain at least one gonadotroph specific element (GSE) to which SF-1 binds to initiate transcription (Bryan *et al.*, 1999, *J. Molec. Endocrin.*, 22:241-249). Preferably, the GSE comprises the sequence RRAGGTCA or YCAAGGYR.

The SF-1 regulated promoter may be derived from the region upstream of any gene the transcription of which is regulated by SF-1. Genes that are linked to an SF-1 regulated promoter in nature from which the SF-1 regulated promoter may be derived include aromatase, lutenising hormone beta, follicle stimulating hormone, StAR, cholesterol side-

chain cleavage enzyme, DAX-1, anti-mullerian hormone, kallikrein and RLF (see Koskimies *et al*, 2002, *Endocrinology* 143(3): 909-919). Mutant SF-1 promoters may also be used *e.g.* those described by Koskimies *et al.* [*supra*].

The reporter gene controlled by the promoter may be a gene which is regulated by SF-1 in nature (see above). In such situations, the reporter construct preferably includes the reporter gene and its natural upstream regulatory sequences.

Preferably, however, the promoter controls transcription of a heterologous reporter gene which is easily assayed, as described above for two-hybrid methods. However, in the two-hybrid system, compounds that inhibit the interaction between SF-1 and Nrip1 are detected as a result of a decrease in the expression of the reporter gene; in contrast, where the transcriptional activity of SF-1 is itself detected, compounds that inhibit the interaction result in an increase in the expression of the reporter gene because repression of SF-1 activity by Nrip1 is inhibited.

For example, where the reporter gene is a fluorescent protein, inhibition of the interaction between Nrip1 and SF-1 may be detected by an increase in fluorescent protein expression (*cf.* two-hybrid system). Where the method is carried out in a cell and the reporter gene is a toxic protein, only cells in which there is no inhibition of the interaction between Nrip1 and SF-1 survive *i.e.* killing is the "positive" result.

The proteins encoded by the reporter genes may be in the form of fusion proteins as described above. For example, genes that give a visible signal may be fused downstream of a gene that is linked to the promoter in the construct in nature.

Indirect screening methods for up-regulation of SF-1 expression:

Screening for compounds that promote the expression of SF-1 may also be conducted indirectly using a reporter gene. In general, such methods operate as described above, with modifications as necessary. Thus the invention provides a method of screening for compounds that promote the expression of SF-1, said method comprising:

- a) contacting a nucleic acid molecule comprising a promoter from a SF-1 gene operatively linked to a reporter gene with one or more candidate compounds; and
- b) assessing the level of expression of the reporter gene.

Compounds that promote SF-1 expression result in increased expression of the reporter

gene.

The promoter is from an SF-1 gene and may be derived from the region upstream of any SF-1 gene. Preferably, the promoter is derived from the region upstream of a mammalian SF-1 gene. The promoter may be derived from the region upstream of the SF-1 gene
5 endogenous to a cell or tissue in which an assay is performed. The promoter may be derived from the region upstream of the human SF-1 gene.

The reporter gene give an easily-assayed product, as described above. Alternatively, the reporter may be SF-1 itself, with the level of SF-1 expression detected indirectly using a nucleic acid molecule comprising a SF-1 regulated promoter operatively linked to a second
10 reporter gene, as described above.

Vectors comprising reporter constructs:

Reporter constructs used indirect screening methods of the invention may be in the form of a viral vector or a non-viral vector. Preferably, the nucleic acid molecules used in these methods of the invention are in the form of a conventional non-viral vector, such as a
15 plasmid. Where these indirect screening methods are conducted in cell-based or tissue-based assays, the introduction of the non-viral vector into the animal cells may be carried out by any method known in the art including dextran-mediated transfection, calcium phosphate precipitation, polybrene-mediated transfection, protoplast fusion, electroporation, encapsulation of polynucleotides in liposomes or direct microinjection of
20 the DNA into nuclei *etc.*.

Use of nucleic acid molecules:

The invention provides the use of a reporter construct, as described above, in a method of screening for anti-ovulatory compounds.

Systems for carrying out screening methods

25 The methods of the invention may be carried out in cell-free systems or in cells or tissues.

In particular, the indirect screening methods described above may be carried out in a cell-free system, in a cell or in a tissue. The cell-free system must contain all the necessary components for transcription of the reporter gene where the level of expression is detected by measuring mRNA levels, and all the necessary components for transcription and
30 translation of the reporter gene where the level of expression is assessed by measuring protein levels. Where the method of screening for compounds that promote expression of

SF-1 is carried out in a cell-free transcription system, for instance, the system must comprise the transcription factors necessary for transcription of the SF-1 gene.

It is preferred that the methods of screening of the invention be conducted in cell-free systems since this facilitates high-throughput screening of candidate compounds.

- 5 Indirect screening methods of the invention are preferably carried out in eukaryotic cells, such as mammalian (*e.g.* human) or yeast cells. They may also be performed in mammalian (*e.g.* human) tissues. A typical cell is an ovary cell or a Leydig cell. A typical tissue is ovarian tissue.

- When the indirect method of screening for compounds that inhibit the interaction of Nrip1 and SF-1 using a nucleic acid molecule comprising an SF-1 regulated promoter is carried
10 out in a cell, the cell should preferably express both Nrip1 and SF-1 endogenously. If Nrip1 and SF-1 are not endogenously expressed, they may be introduced into the cell using a viral or non-viral vector encoding the Nrip1 or SF-1. Preferably, the Nrip1 and SF-1 are introduced into the cell in the form of plasmids.

15 **Assessing level of expression of reporter gene:**

The level of expression of the reporter gene may be assessed by measuring the level of a mRNA transcribed from the reporter gene or the level of protein translated after its transcription.

Measuring level of mRNA

- 20 The level of mRNA transcribed from a reporter gene can be assessed, for example, by traditional blotting techniques described in Sambrook *et al* [*supra*]. Messenger RNA can be purified and separated using gel electrophoresis. The nucleic acids on the gel are then blotted onto a solid support, such as nitrocellulose. The solid support is exposed to a labelled probe and then washed to remove any unhybridized probe. Next, the duplexes
25 containing the labelled probe are detected. Typically, the probe is labelled with a radioactive moiety.

- Alternatively, the level of mRNA transcribed from the reporter gene may be detected by PCR-based methods. The mRNA transcribed from the reporter gene may be specifically amplified using primers that only bind to the mRNA with the amplified mRNA being
30 detected using the blotting methods described above. The level of transcription of the reporter gene could be also detected using fluorescence resonance energy transfer (FRET)

through fluorophores coupled to two oligonucleotides that are complementary to the mRNA transcribed from the reporter gene. (see Wouters *et al*, 2001, *Trends in Cell Biology* 11, 203-211).

- As the cell-free system, cell or tissue will contain DNA from which the mRNA is transcribed, it is preferred to use a RNA-specific detection technique or to focus on sequence present in the mRNA transcription but not in the DNA (*e.g.* splice junctions, polyA tail *etc.*). The methods of the invention may comprise an initial step of: extracting mRNA from the cell-free system, cell or tissue; removing DNA from the cell-free system, cell or tissue; and/or disrupting DNA but not mRNA in the cell-free system, cell or tissue.
- 10 Methods for selectively extracting RNA from biological samples are well known and include methods based on guanidinium buffers, lithium chloride, acid phenol:chloroform extraction, SDS/potassium acetate *etc.* After total RNA has been extracted, mRNA may be enriched for example using oligo-dT techniques. Methods for removing DNA from biological samples include DNase digestion. Methods for removing DNA encoding the
- 15 reporter gene transcript but not the RNA transcribed from it will use an agent which is specific to a sequence within the DNA.

Measuring level of protein

- Measurement of mRNA levels is not ideal in high throughput screening methods. The invention also provides that reporter gene expression can be assessed by measuring protein
- 20 levels.

- The level of protein expressed from the reporter gene can be conveniently measured by using an antibody which binds to the protein encoded by the reporter gene. Following removal of unbound antibody, the level of the protein encoded by the reporter gene can be determined by assessing the level of the antibody bound to it. This may be done by
- 25 labelling the antibody that binds to the protein or by using a second labelled antibody which binds to the first antibody.

- Where the reporter gene encodes a protein that provides a visible signal, the level of expression of the reporter gene is preferably assessed by detecting the visible signal. For example, where the reporter gene encodes a fluorescent protein such as GFP, or an enzyme
- 30 such as luciferase, the level of expression may be assessed by fluorescence/luminescence detection. Where the reporter gene encodes a protein that is toxic or cytostatic, the level of expression may be assessed by looking at cell survival or cell growth.

Reference standards:

A reference standard (*e.g.* a control), is typically needed in order to detect whether the interaction between SF-1 and Nrip1 is inhibited in the first method of the invention and whether the expression of SF-1 is up-regulated in the second method. In order to detect
5 whether a candidate compound inhibits the interaction between SF-1 and Nrip1, the interaction between SF-1 and Nrip1 in the presence of a candidate compound may be compared with the interaction between SF-1 and Nrip1 in the absence of a candidate compound. In order to detect whether the expression of SF-1 is promoted by a candidate compound, the level of expression of SF-1 in the presence of a candidate compound may
10 be compared with the level of expression of SF-1 in the absence of the candidate compound.

The reference may have been determined before performing the method of the invention, or may be determined during (*e.g.* in parallel) or after the method has been performed. It may be an absolute standard derived from previous work.

15 SF-1 and Nrip1:

The methods of the invention may use SF-1 and Nrip1 derived from any eukaryote. Preferably, they use SF-1 and Nrip1 derived from an animal, such as a mammal.

The SF-1 gene has been cloned and sequenced in a number of mammalian species including mouse (Tsukiyama T *et al*, 1992, *Mol Cell Biol* 12:1286-1291; Ikeda Y *et al*,
20 1993, *Mol Endocrin*, 7: 852-860) and sheep (NCBI database entry AAG24622.1 GI:10945629). The human gene for the transcription factor SF-1 has also been cloned and sequenced (Wong *et al*, 1996, *J. Mol Endocrinol*, 17(2): 139-47) and its protein structure has been studied (Oba *et al*, 1996, *Biochem Biophys Res Commun*, 226(1): 261-7). The SF-1 gene is evolutionarily conserved in vertebrates and invertebrates and homologues of the
25 vertebrate genes are found in a number of invertebrates including *Drosophila* and hookworm (see Parker & Schimmer, 1997, *Endocrine Reviews* 18(3): 361-376 for review).

The Nrip1 gene has been cloned in a number of mammalian species including humans (Cavaillès *et al*, 1995, *EMBO J.*, 14:3741-3751) and mouse (Lee *et al*, 1998, *Mol Cell Biol*, 18: 6745-55).

30 Preferably, Nrip1 and SF-1 used in a method of the invention are both derived from the same mammal. The Nrip1 and SF-1 proteins are preferably both human proteins and, when SF-1-regulated reporter constructs are used, the promoter is preferably a human promoter.

Reference in the methods of the invention to the use of SF-1 and Nrip1 includes the use of variants of SF-1 which retain the ability to interact with Nrip1, and *vice versa*. Suitable variants are known from the literature *e.g.* a number of human SF-1 protein variants are known to be generated by alternative splicing (Kramer *et al*, 1998, *Gene*, 211(1): 29-37) and these variants may be used in the methods of the invention. Other variants include natural biological variants, allelic variants, fragments, and mutants containing amino acid substitutions, insertions or deletions from the wild type sequence.

Fragments of natural SF-1 that interact with Nrip1 and fragments of Nrip1 that interact with SF-1 may also be used in the methods of the invention. Motifs in Nrip1 that interact with nuclear receptors are disclosed in WO98/49561, Lee *et al* (*Mol Cell Biol*, 1998, 18(11): 6745-44) and in Wei *et al* (*J. Biol. Chem.*, 2001, 276(19): 16107-12). References to the use of SF-1 and Nrip1 in the methods of the invention also includes the use of such fragments of SF-1 and Nrip1.

Polypeptides that are structurally similar to SF-1 and Nrip1, or to fragments of Nrip1 and SF-1 that retain the ability to interact, may also be used in the methods of the invention. These may be derived from natural SF-1 or Nrip1 or they may be prepared synthetically or using techniques of genetic engineering. In particular, synthetic molecules that are designed to mimic the tertiary structure of SF-1 or Nrip1 and in particular the domains of SF-1 and Nrip1 that interact may be used in the methods of the invention. References to the use of SF-1 and Nrip1 in the methods of the invention include the use of polypeptides that are structurally similar to SF-1 and Nrip1, or to fragments thereof.

References to the use SF-1 and Nrip1 in the methods of the invention also include the use of fusion proteins comprising SF-1 or Nrip1, fusion proteins comprising variants or fragments thereof, or fusion proteins comprising polypeptides that are structurally similar to SF-1 or Nrip1 or to fragments of SF-1 or Nrip1. Such fusion proteins are particularly useful in two-hybrid methods.

Candidate compounds

Candidate compounds used in screening methods:

Typical candidate compounds for use in all the screening methods of the invention include, but are not restricted to, peptides, peptoids, proteins, lipids, metals, small organic molecules, RNA aptamers, antibiotics and other known pharmaceuticals, polyamines, antibodies or antibody derivatives (*e.g.* antigen-binding fragments, single chain antibodies

including scFvs, etc), and combinations or derivatives thereof. Small organic molecules have a molecular weight of about more than 50 and less than about 2,500 daltons, and most preferably between about 300 and about 800 daltons. Candidate compounds may be derived from large libraries of synthetic or natural compounds. For instance, synthetic
5 compound libraries are commercially available from MayBridge Chemical Co. (Revillet, Cornwall, UK) or Aldrich (Milwaukee, WI). Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts may be used. Additionally, candidate compounds may be synthetically produced using combinatorial chemistry either as individual compounds or as mixtures.

10 Preliminary screening for candidate compounds:

In some instances, it may be desirable to conduct a preliminary screening step to reduce the number of candidate compounds used in the methods of the invention. Compounds that bind to Nrip1 or SF-1 individually are inherently more likely to inhibit interaction between SF-1 and Nrip1 than those that do not.

- 15 The invention therefore provides a method of screening for compounds that inhibit the interaction between Nrip1 and SF-1 further comprising the preliminary step of screening for candidate compounds by identifying compounds that bind to either Nrip1 or SF-1.

Candidate compounds may be pre-screened in this way using the two-hybrid systems. In particular, candidate compounds may be compounds identified as binding to the signature
20 motif in Nrip1 identified in WO98/49561, Lee *et al* (*Mol Cell Biol*, 1998, 18(11): 6745-44) or in Wei *et al* (*J. Biol. Chem.*, 2001, 276(19): 16107-12). Pre-screening may also be used with antibodies (or derivatives).

It has been suggested that the binding of Nrip1 to nuclear receptors may be inhibited by acetylation of the domains in Nrip1 that interacts with the receptor (Vo *et al*, 2001, *Mol*
25 *Cell Biol*, 21(18): 6181-8). Candidate compounds may therefore be compounds identified as acetylating Nrip1 in a pre-screen.

Candidate compounds may also be compounds identified in a pre-screen as binding to the carboxy-terminal transcriptional activation domain of SF-1 (Ito *et al*, 1998, *Mol. Endocrinol.*, 12(2):290-301).

In vivo confirmation of function of compounds identified

Once a compound has been identified as an inhibitor of the interaction between SF-1 and Nrip1 or as a promoter of the expression of SF-1, it may be desirable to perform further experiments to confirm the *in vivo* anti-ovulatory function of the compound.

- 5 The invention therefore provides a method of assessing the anti-ovulatory effect of a compound obtained or obtainable by any of the methods described above in vivo comprising administering the compound to a female mammal and assessing the effect on ovulation. The female mammal may be any species of mammal but is preferably a monkey, pig, rabbit or mouse. Tests in non-humans may be used.
- 10 The methods of screening for compounds that inhibit the interaction between SF-1 and Nrip1 or that promote the expression of SF-1 may take place in a different geographical location from the method for assessing the anti-ovulatory effect of the compounds identified.

Compounds identified by screening methods

- 15 The invention further provides a compound that inhibits the interaction between Nrip1 and SF-1 obtained or obtainable by any of the methods described above. The invention also provides a compound that promotes expression of SF-1 obtained or obtainable by any of the methods described above. Preferably, the compounds of the invention are organic compounds.
- 20 There is also provided a composition comprising a compound that inhibits the interaction between Nrip1 and SF-1 or a compound that promotes expression of SF-1, obtained or obtainable by any of the methods described above.

- Compounds that are found to inhibit ovulation may be useful contraceptives in their own right or may be lead compounds for the development of new contraceptives. They may
- 25 also be useful in contraceptive research.

Pharmaceutical uses of compounds identified

- Once a compound has been identified using one of the methods of the invention, it may be necessary to conduct further work on its pharmaceutical properties. For example, it may be necessary to alter the compound to improve its pharmacokinetic properties or
- 30 bioavailability. The invention extends to any compounds identified by the methods of the

invention which have been altered to improve their pharmacokinetic properties, and to composition comprising those compounds.

The invention further provides compounds obtained or obtainable using the methods of the invention, and compositions comprising those compounds, for use as contraceptives. The
5 invention also provides the use of a compounds obtained or obtainable using the methods of the invention, or compositions comprising those compounds in the manufacture of a medicament to prevent ovulation. A contraceptive method comprising administering a compound obtained or obtainable by any one of the methods of the invention, or a composition comprising such a compound, to a mammal, preferably a human, is also
10 provided.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows kallikrein (KLK) expression analysis in murine wild-type and Nrip knock-out ovarian samples. Figure 1A shows a Northern blot. Figure 1B shows real-time PCR analysis of tissue KLK, with clear up-regulation in homozygous knockout mice. Figure 1C
15 shows *in situ* staining of wild-type tissue and Figure 1D shows the same staining for a homozygous knockout.

Figure 2 shows TaqMan real-time PCR analysis of RLF/INSL3 expression in ovarian samples.

Figure 3 shows SF-1 regulated expression of TKLK and RLF/INSL3 in transiently
20 transfected 293 cells.

EXAMPLES:

Various aspects and embodiments of the present invention will now be described in some detail. It will be appreciated that modification of detail may be made without departing from the spirit and scope of the invention.

25 Kallikrein expression analysis in wild-type and Nrip knock-out ovarian samples.

Northern blot, TaqMan real-time PCR and In Situ hybridisation analysis were used to detect the expression pattern of the kallikrein gene in Nrip1 knock-out mice. As shown in Figure 1, Northern Blot analysis (1A) could demonstrate kallikrein (KLK) expression only in Nrip1 knock-out RNA samples. TaqMan real-time analysis (1B) showed that the mRNA
30 levels of KLK during the ovulatory process is dramatically elevated in immature and to an even higher degree in mature knock-out mice compared to wild-type and heterozygous

mice. In Situ hybridisation analysis (1C & 1D) showed that kallikreins are expressed in ovarian theca/interstitial cells.

TaqMan real-time PCR analysis of RLF/INSL3 expression in ovarian samples.

As shown in Figure 2, TaqMan real-time PCR analysis demonstrated that, as for
5 kallikreins, the mRNA level of RLF/INSL3 is dramatically elevated in KO mice (red) compared to wild-type and heterozygous mice.

Transient transfection analysis of kallikrein and RLF/INSL3 promoter activity.

The mouse RLF gene contains several binding sites for SF-1, which have been found to be functionally important in transient transfections of leydig (mLTC) and luteinized ovary
10 (KK1) cells (Koskimies *et al*, 2002, *Endocrinol.*, 143(3): 909-919). When the mouse kallikrein promoter was analysed, it was also found to contain several potential SF-1 binding sites.

To determine whether SF-1 could regulate expression of RLF and KLK from their respective promoters and to what extent any expression was modulated by Nrip1, transient
15 transfections of 293 cells with luciferase reporter constructs containing DNA fragments of the respective promoters were performed. A transient transfection of 293 cells with an empty reporter vector (pGL3-basic) was also performed. Expression plasmids containing SF-1 and Nrip1 were co-transfected as indicated in Figure 3.

As shown in Figure 3, expression from both the KLK and RLF/INSL3 constructs can be
20 activated with co-transfection of SF-1. However, addition of Nrip1 essentially represses all SF-1 mediated up-regulation, suggesting that Nrip1 has the potential regulate SF-1 mediated expression. Lack of Nrip1 activity in Nrip1 KO mice may therefore result in over-expression of SF-1 regulated genes *in vivo*.

The reporter constructs comprising an SF-1 regulated promoter linked to a luciferase
25 reporter gene can be used to screen for candidate compounds that reverse the inhibition of SF-1 by Nrip1. Figure 3 shows the level of expression of the reporter gene in the presence of Nrip1 and SF-1. The experiments described above can be repeated in the presence of a candidate compound to identify compounds that increase the level of expression of the reporter gene. Candidate compounds that increase the level of expression of the reporter
30 gene may have anti-ovulatory properties.

Further experimental details can be found in Parker *et al.* (2003) *FEBS Lett* 546:149-153.

CLAIMS

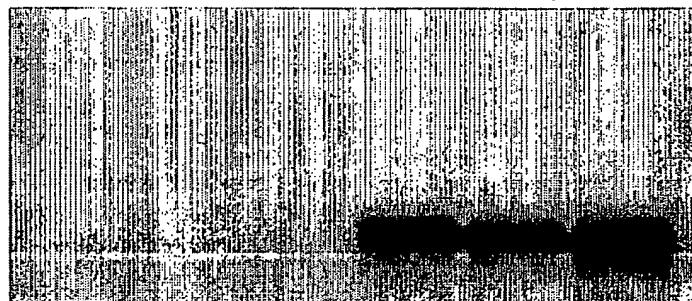
1. A method of screening for compounds that inhibit the interaction between SF-1 and Nrip1, said method comprising assessing inhibition of the interaction between SF-1 and Nrip1 in the presence of one or more candidate compounds.
- 5 2. A method according to claim 1 comprising:
 - a) mixing Nrip1, SF-1 and one or more candidate compounds;
 - b) incubating the mixture to allow Nrip1, SF-1 and the candidate compound(s) to interact; and
 - c) assessing whether interaction between Nrip1 and SF-1 is inhibited.
- 10 3. A method according to claim 1 comprising:
 - a) contacting a cell containing a nucleic acid molecule comprising a promoter operatively linked to a reporter gene with: (i) a first fusion protein comprising one of SF-1 and Nrip1 fused to the activation domain of a transcription factor, (ii) a second fusion protein comprising the other of SF-1 and Nrip1 fused to the
15 DNA-binding domain of a transcription factor; and (iii) a candidate compound; and
 - b) assessing the level of expression of the reporter gene,wherein interaction between SF-1 and Nrip1 promotes transcription of the reporter gene by activating said promoter.
- 20 4. A method according to claim 1 comprising:
 - a) contacting a nucleic acid molecule, comprising an SF-1 regulated promoter operatively linked to a reporter gene, with one or more candidate compound(s) in the presence of SF-1 and Nrip1; and
 - b) assessing the level of expression of the reporter gene.
- 25 5. A method of screening for compounds that up-regulate expression of SF-1, said method comprising:
 - a) contacting a nucleic acid molecule comprising a promoter from a SF-1 gene operatively linked to a reporter gene with a candidate compound; and
 - b) assessing the level of expression of the reporter gene.
- 30 6. A method according to any one of claims 3 to 5, wherein the promoter controls transcription of a reporter gene with which it is linked in nature.

7. A method according to any one of claims 3 to 6, wherein expression of the reporter gene gives a detectable signal.
8. A method according to claim 7, wherein the reporter gene encodes a fluorescent protein or an enzyme.
- 5 9. A reporter gene according to claim 7, wherein the reporter gene encodes a toxic or cystostatic protein.
10. A method according to any preceding claim, wherein said method is carried out in a cell free system, a cell or a tissue.
11. A method according to any one of claims 3 to 10, wherein the nucleic acid molecule is
10 in the form of a non-viral vector.
12. A method according to any one of claims 3 to 11, wherein the step of assessing the level of expression of the reporter gene comprises measuring the level of mRNA transcribed from the reporter gene.
13. A method according to any one of claims 3 to 11, wherein the step of assessing the
15 level of expression of the reporter gene comprises measuring the level of protein translated after transcription of the reporter gene.
14. A method according to any preceding claim, comprising the further steps of administering to a female mammal a candidate compound found to inhibit the interaction between SF-1 and Nrip1, or to up-regulate SF-1 expression, and assessing
20 its effect on ovulation.
15. A compound that inhibits the interaction between Nrip1 and SF-1, obtained or obtainable by a method of any one claims 1 to 4 or 6 to 13.
16. A compound that up-regulated expression of SF-1, obtained or obtainable by a method of any one of claims 5 to 13.
- 25 17. A method of assessing the anti-ovulatory effect of a compound according to claim 15 or claim 16, comprising administering the compound to a female mammal and assessing its effect on ovulation.
18. A composition comprising a compound according to claim 15 or claim 16.
19. A compound according to claim 15 or claim 16 for use as a contraceptive.

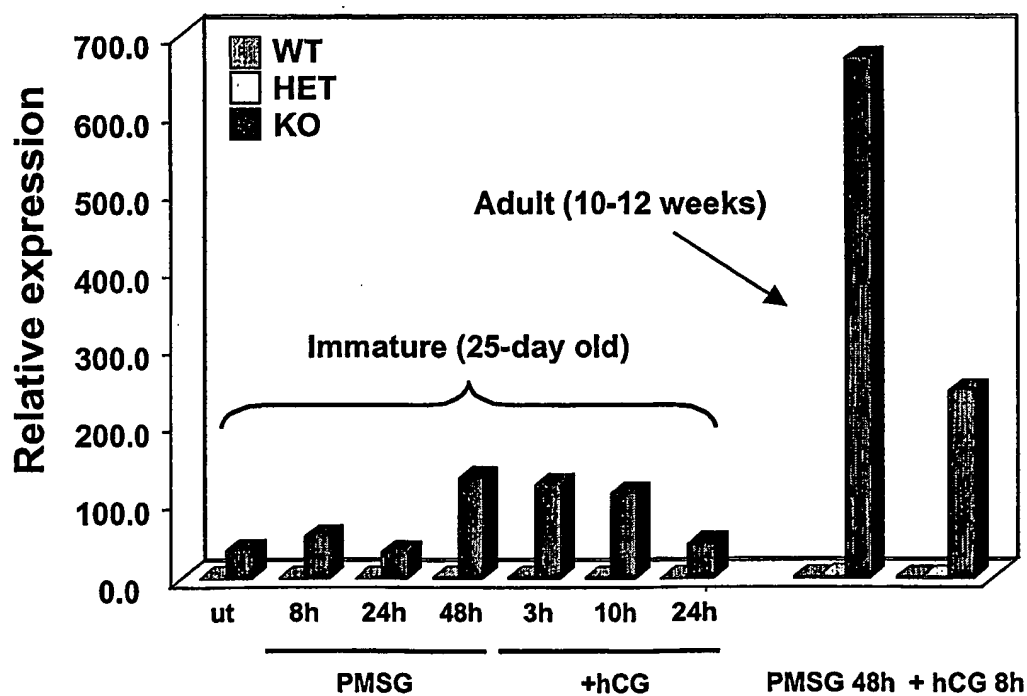
20. Use of a compound according to claim 15 or claim 16 in the manufacture of a medicament to prevent ovulation.
21. A contraceptive method comprising administering a compound according to any claim 15 or claim 16, or a composition according to claim 18, to female mammal.

FIGURE 1A**10/519404**

wt wt wt ko ko ko



← 18S

FIGURE 1B

10/519404

FIGURE 1C

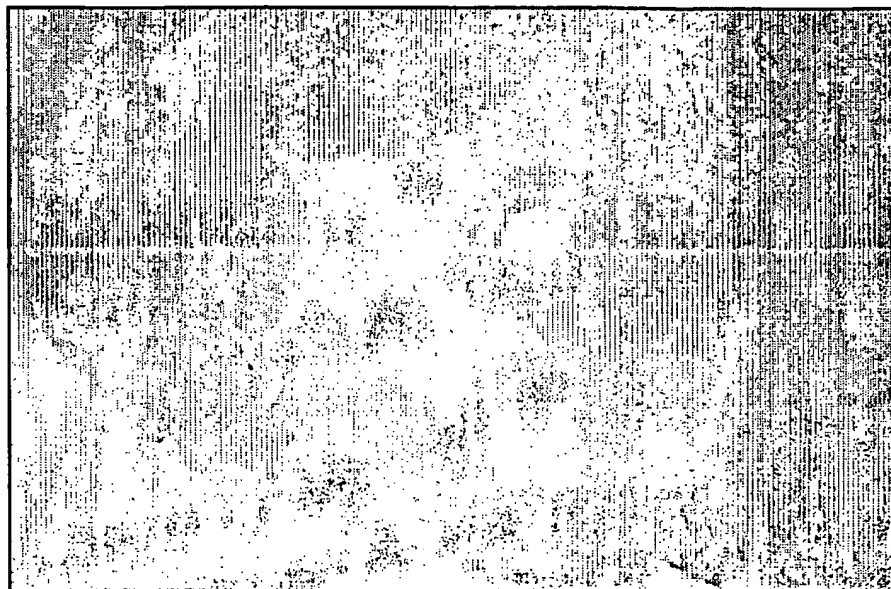
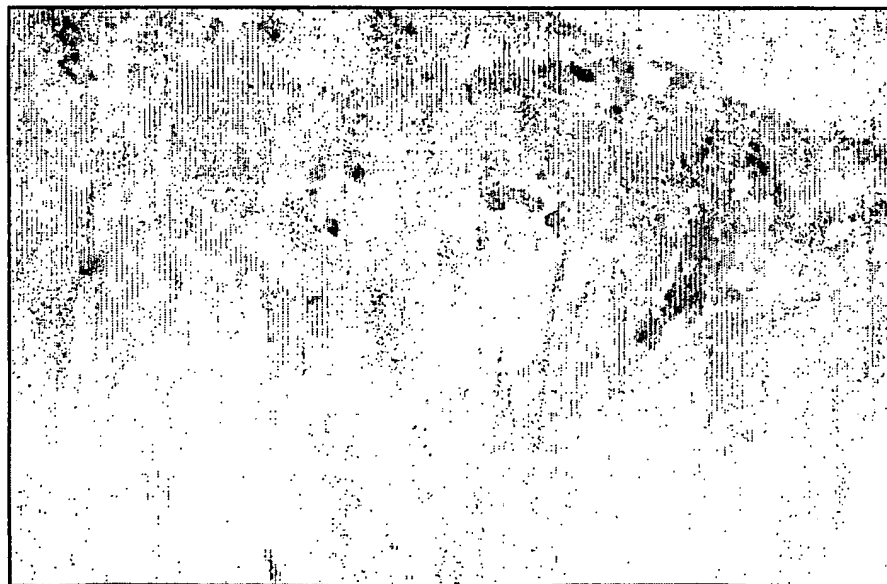
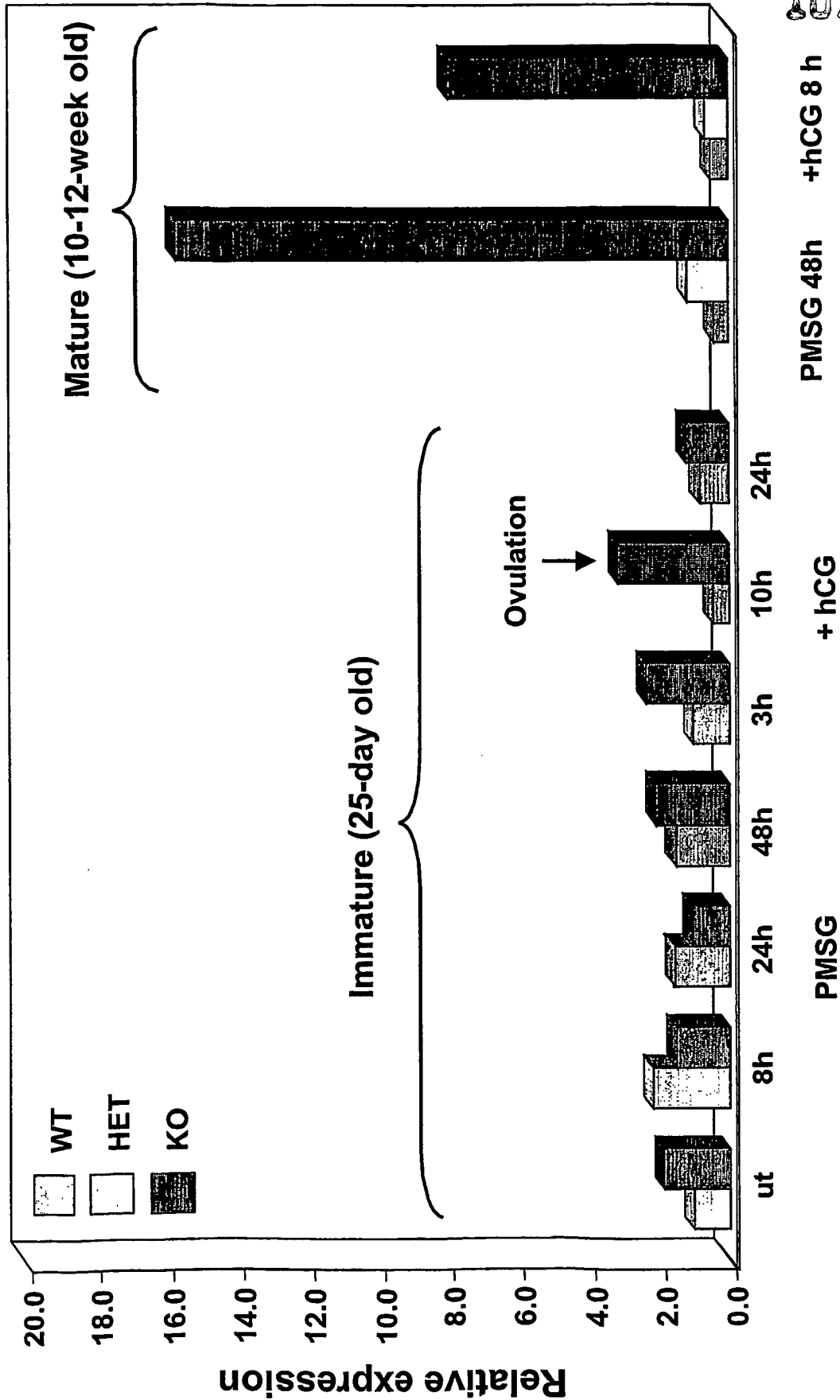


FIGURE 1D

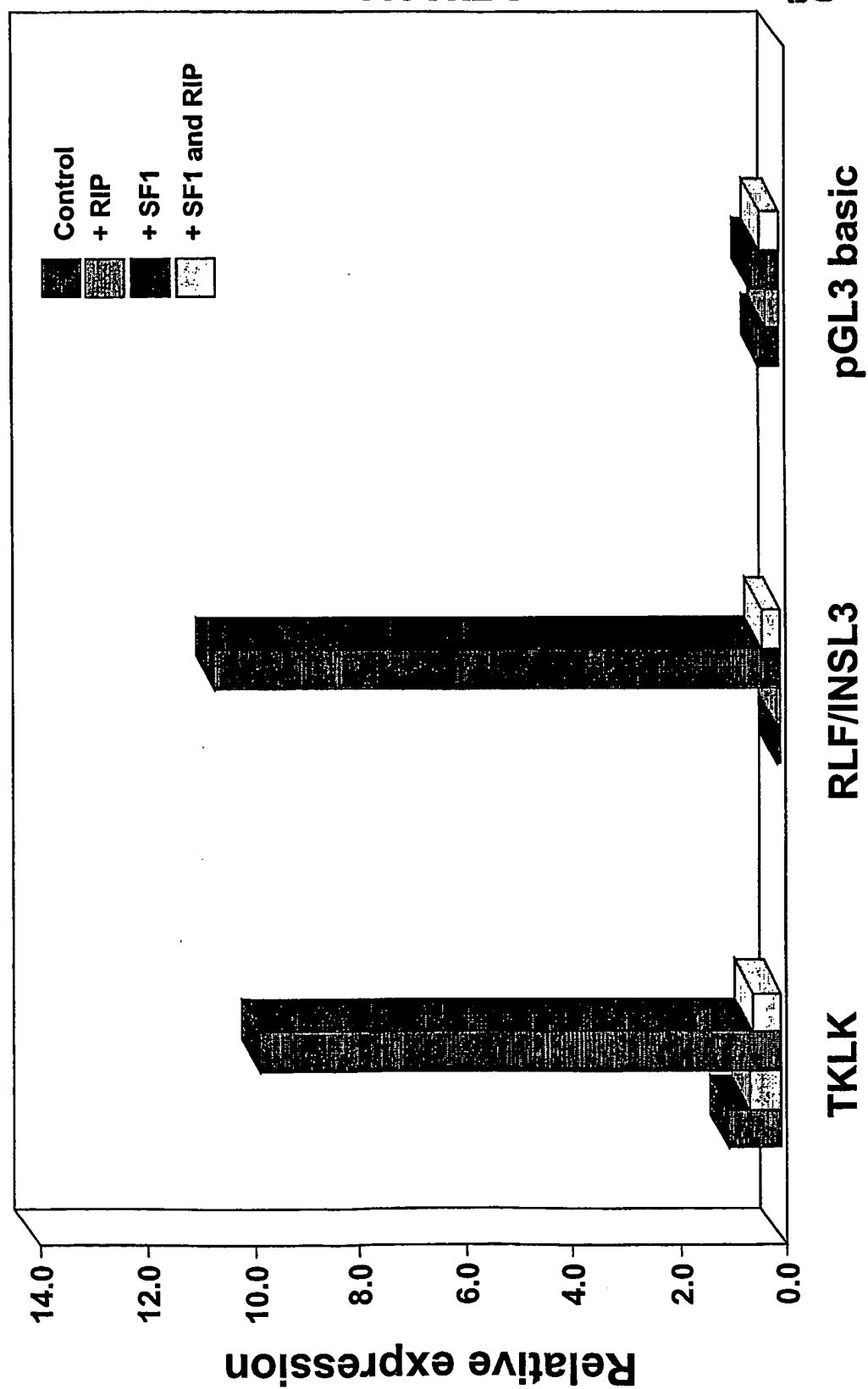


10/519404

FIGURE 2



10/519404

FIGURE 3

INTERNATIONAL SEARCH REPORT

International Application No

PCT/JP 03/02876

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

MEDLINE, EMBASE, BIOSIS, CHEM ABS Data, EPO-Internal, WPI Data, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>MELLGREN ET AL.: "Receptor Interacting Protein RIP140 inhibits transcriptional activity of steroidogenic factor-1" ENDO, - June 2001 (2001-06) page 541 XP008023461 83rd Annual Meeting Denver, Colorado: Program and Abstracts. Bethesda, MD, USA: Endocrine Society Press; Endo 2001. Denver, USA, 20-23. Jun 2001 the whole document</p> <p style="text-align: center;">-/--</p>	1-4, 6-8, 10-13

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

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Date of the actual completion of the international search

24 October 2003

Date of mailing of the international search report

10/11/2003

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Hagenmaier, S

INTERNATIONAL SEARCH REPORT

 Internatl Application No
 PCT/US 03/02876

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>SUGAWARA T ET AL: "RIP 140 modulates transcription of the steroidogenic acute regulatory protein gene through interactions with both SF-1 and DAX-1." ENDOCRINOLOGY. UNITED STATES AUG 2001, vol. 142, no. 8, August 2001 (2001-08), pages 3570-3577, XP002258562 ISSN: 0013-7227 cited in the application the whole document</p>	1-4,6-13
X	<p>SHEN JENNIFER H-C ET AL: "Regulation of the orphan nuclear receptor steroidogenic factor 1 by Sox proteins." MOLECULAR ENDOCRINOLOGY (BALTIMORE, MD.) UNITED STATES MAR 2002, vol. 16, no. 3, March 2002 (2002-03), pages 529-540, XP002258564 ISSN: 0888-8809 the whole document</p>	5-8, 10-13
X	<p>EMMEN J M ET AL: "Involvement of insulin-like factor 3 (Ins13) in diethylstilbestrol-induced cryptorchidism." ENDOCRINOLOGY. UNITED STATES FEB 2000, vol. 141, no. 2, February 2000 (2000-02), pages 846-849, XP002258563 ISSN: 0013-7227 the whole document</p>	5-8, 10-13
X	<p>WO 98 37207 A (HICKSON IAN DAVID ;IMP CANCER RES TECH (GB); EDWARDS SUSAN NICOLA) 27 August 1998 (1998-08-27) the whole document</p>	9
X	<p>DETRAIT ERIC R ET AL: "Reporter gene transfer induces apoptosis in primary cortical neurons." MOLECULAR THERAPY: THE JOURNAL OF THE AMERICAN SOCIETY OF GENE THERAPY. UNITED STATES JUN 2002, vol. 5, no. 6, June 2002 (2002-06), pages 723-730, XP002258886 ISSN: 1525-0016 the whole document</p>	9
Y	<p>PARKER K L ET AL: "The roles of steroidogenic factor-1 in reproductive function." STEROIDS. UNITED STATES APR 1996, vol. 61, no. 4, April 1996 (1996-04), pages 161-165, XP002258565 ISSN: 0039-128X the whole document</p>	5-8, 10-13
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INTERNATIONAL SEARCH REPORT

International Application No

PCT/JP 03/02876

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CARON K M ET AL: "Steroidogenic factor 1 acts at all levels of the reproductive axis." STEROIDS. UNITED STATES JAN 1997, vol. 62, no. 1, January 1997 (1997-01), pages 53-56, XP002258566 ISSN: 0039-128X the whole document ---	5-8, 10-13
Y	PARKER K L ET AL: "Steroidogenic factor 1: a key determinant of endocrine development and function." ENDOCRINE REVIEWS. UNITED STATES JUN 1997, vol. 18, no. 3, June 1997 (1997-06), pages 361-377, XP008023428 ISSN: 0163-769X the whole document ---	5-8, 10-13
Y	BORUD BENTE ET AL: "The nuclear receptor coactivators p300/CBP/cointegrator-associated protein (p/CIP) and transcription intermediary factor 2 (TIF2) differentially regulate PKA-stimulated transcriptional activity of steroidogenic factor 1." MOLECULAR ENDOCRINOLOGY (BALTIMORE, MD.) UNITED STATES APR 2002, vol. 16, no. 4, April 2002 (2002-04), pages 757-773, XP002258769 ISSN: 0888-8809 the whole document ---	5-8, 10-13
A	WHITE R ET AL: "The nuclear receptor co-repressor nr1p1 (RIP140) is essential for female fertility." NATURE MEDICINE. UNITED STATES DEC 2000, vol. 6, no. 12, December 2000 (2000-12), pages 1368-1374, XP002258567 ISSN: 1078-8956 cited in the application the whole document ---	
A	LEONARDSSON GÖRAN ET AL: "Embryo transfer experiments and ovarian transplantation identify the ovary as the only site in which nuclear receptor interacting protein 1/RIP140 action is crucial for female fertility." ENDOCRINOLOGY. UNITED STATES FEB 2002, vol. 143, no. 2, February 2002 (2002-02), pages 700-707, XP002258568 ISSN: 0013-7227 cited in the application the whole document ---	
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INTERNATIONAL SEARCH REPORT

 Internat .pplication No
 PCT/GB J3/02876

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 98 49561 A (HEERY DAVID MICHAEL ;IMP CANCER RES TECH (GB); PARKER MALCOLM GEOR) 5 November 1998 (1998-11-05) cited in the application the whole document ----	
A	WO 02 16426 A (AKZO NOBEL NV ;DECHERING KOEN JACOB (NL); MOSSELMAN SIETSE (NL)) 28 February 2002 (2002-02-28) the whole document ----	
A	FIELDS S ET AL: "THE TWO-HYBRID SYSTEM: AN ASSAY FOR PROTEIN-PROTEIN INTERACTIONS" FALSTROM FLEX-I-TORCH FOR TIG ARC WELDING, XX, XX, vol. 10, no. 8, August 1994 (1994-08), pages 286-292, XP002937846 the whole document ----	
P,X	MELLGREN GUNNAR ET AL: "Characterization of receptor-interacting protein RIP140 in the regulation of SF-1 responsive target genes." MOLECULAR AND CELLULAR ENDOCRINOLOGY. IRELAND 30 MAY 2003, vol. 203, no. 1-2, 30 May 2003 (2003-05-30), pages 91-103, XP002258569 ISSN: 0303-7207 the whole document ----	1-4,6-13
P,A	PARKER MALCOLM ET AL: "Identification of RIP140 as a nuclear receptor cofactor with a role in female reproduction." FEBS LETTERS. NETHERLANDS 3 JUL 2003, vol. 546, no. 1, 3 July 2003 (2003-07-03), pages 149-153, XP002258570 ISSN: 0014-5793 the whole document -----	

INTERNATIONAL SEARCH REPORT

International application No.
T/GB 03/02876

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 14, 17, 21
because they relate to subject matter not required to be searched by this Authority, namely:
Rule 39.1(iv) PCT - Method for treatment of the human or animal body by therapy
2. ☒ Claims Nos.: 14-21
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this International application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-4 (all completely), 6-8,10-13 (all partially)

A method of screening for compounds that inhibit the interaction between SF-1 and NRIP1, said method comprising assessing inhibition of the interaction between SF-1 and NRIP1 in the presence of one or more candidate compounds.

2. Claims: 5 (completely), 6-8, 10-13 (all partially)

A method of screening for compounds that upregulate expression of SF-1, said method comprises:
a) contacting a nucleic acid molecule comprising a promoter from a SF-1 gene operatively linked to a reporter gene with a candidate compound; and
b) assessing the level of expression of the reporter gene.

3. Claim : 9 (completely)

Reporter gene which encodes a toxic or cytostatic protein.

4. Claims: 15 (completely), 14,17-21 (all partially)

A compound that inhibits the interaction between SF-1 and NRIP1 and methods using such a compound.

5. Claims: 16 (completely),14,17-21 (all partially)

A compound that up-regulates the expression of SF-1 and methods using such a compound.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 14-21

Present claims 14-21 relate to compounds defined by reference to a desirable characteristic or property, namely a compound which inhibit the interaction between Nrip1 and SF-1 or a compound which up-regulates SF-1 expression. The claims cover all compounds having this characteristic or property, whereas the application provides support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT for no such compounds. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An attempt is made to define the compounds by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search impossible. Consequently, no search has been carried out for these claims.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

 International Application No
 PCT/GB 03/02876

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
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WO 0216426	A	28-02-2002	AU 1214202 A CA 2419981 A1 WO 0216426 A2 EP 1313766 A2	04-03-2002 28-02-2002 28-02-2002 28-05-2003

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